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Award Number: DAMD17-00-1-0168

TITLE: Differential Expression of DNA Double-Strand Break Repair Proteins in Breast Cells

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (1 Jul 2000 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Differential Expression of DNA Double-Strand Break Repair Proteins in Breast Cells

5. FUNDING NUMBERS
DAMD17-00-1-0168

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Two mechanisms that repair DNA double-strand breaks in mammalian cells are homologous recombination and non-homologous DNA end-joining (NHEJ). Previous studies showed that a critical component of the NHEJ pathway, the DNA-activated protein kinase (DNA-PK), was poorly expressed in non-lactating (resting) breast tissue. Therefore, we proposed to identify the mechanisms responsible for regulating levels of non-homologous end-joining DNA repair components in human breast tissue and to measure the DNA double-strand break repair capacity of breast epithelial cells.

We reexamined the expression of DNA-PK in human breast tissues by immuno-histochemistry and extended these studies to two other components of the NHEJ repair pathway, XRCC4 and DNA ligase IV, as well as other DNA repair components including NBS1 and MRE11. In contrast to the original report, 90% of the epithelial cells in normal resting breast tissues from 10 different patients expressed both components of DNA-PK, DNAPKcs and Ku. In contrast, stromal cells failed to express NHEJ proteins, but a cell line derived from breast stromal tissue did. No polymorphisms were detected in the Ku70 gene of 14 breast cancer patients, but a high frequency fragment length polymorhism was identified in the promoter region of the Ku80 gene from breast cancer patients. We also showed that 11.3% of breast cancer patients amplified the gene for the Wip1 phosphatase that regulates p53 activity. Furthermore, mouse cells lacking the Wip1 gene were resistant to cell transformation, and mice lacking the Wip1 gene were resistant to tumor formation, suggesting that Wip1 is a potential target for anti-cancer drugs.

14. SUBJECT TERMS
Gene expression, epithelial cells, cell differentiation, DNA repair

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

DNA double-strand break (DSB) repair is critical for cell survival and for preventing genome rearrangements leading to cancer. It is carried out by two mechanisms, homologous recombination and non-homologous DNA end joining (NHEJ). NHEJ is the major repair pathway of DNA repair in mammalian cells and is mediated by three complexes: the DNA-PK complex (DNA-PKcs, Ku70, Ku80), the XRCC4/DNA Ligase IV complex and the NBS1/Rad50/MRE11 complex. We recently observed that DNA-activated protein kinase (DNA-PKcs), a critical component of the NHEJ repair pathway in vertebrates, was poorly expressed in non-lactating (resting) human breast tissue compared to lactating breast tissue, where it was expressed at high levels. All tissues exhibited similar amounts of mRNAs for the three polypeptide components that comprise DNA-PK, suggesting that the poor expression of DNA-PKcs in resting breast epithelial cells results from posttranscriptional mechanisms rather than transcriptional regulation. Our findings suggested that resting breast tissue might be compromised for the repair of DSBs compared to other tissues. Therefore, we proposed to measure the DNA double-strand break repair capacity of breast cells and identify the mechanisms responsible for regulating levels of NHEJ DNA repair components in human breast tissue.

To accomplish these goals, we proposed to: I) establish culture conditions that recapitulate DNA-PK and NHEJ component expression in breast tissue; II) examine the effects of low and high expression levels on DNA repair and genome stability; III) identify the mechanisms that regulate NHEJ component expression in breast tissue.

Body

The objectives of the project were based on the findings (Moll et al., 1999) that epithelial cells in lactating human breast tissue expressed high levels of both DNA-PKcs and Ku proteins while none of these proteins were detected in epithelial cells in normal (resting) human breast tissue. However (as stated in last annual report), when these findings were recapitulated on a larger scale, both resting as well as lactating breast tissue sections of normal women showed strong expression of DNA-PKcs, Ku70, Ku80, XRCC4, and DNA ligase IV as well as NBS1 and MRE11. The interesting observation emerging from our analysis was that stromal cells in these breast tissue sections were devoid of any of these proteins or expressed them at very low levels. Micrographs of breast tissue sections stained with antibodies to DNA-PKcs and other DNA repair proteins are shown in Figure 1 (Appendix).

To investigate the expression of NHEJ proteins in stromal cells from breast tissue in more detail, a representative stromal cell line was identified and obtained from the ATCC (cat. # CRL-7345). This cell line, Hs574.T, which is of fibroblast type, was derived from the ductal carcinoma of a human mammary gland and apparently is of stromal origin. The cell line is from the Naval Biosciences Laboratory collection that was transferred to the ATTC in 1982. A primary stromal cell line, RMF.2 (Elenbaas et al., 2001), derived from fibroblasts isolated from

the normal breast tissue of a reduction mammoplasty sample, also was obtained from Dr. R. Weinberg's lab.

Expression of DNA-PKcs and other NHEJ repair proteins has been studied in Hs 574.T and RMF.2 cells by immunofluorescence (IF) and Western immunoblot assay using available antibodies specific for each NHEJ protein. The expression of DNA-PKcs and both Ku proteins were found to be comparable to the expression in other human cell lines including HeLa and MO59K (Appendix, Figure 2). The DNA-PK activity in these cells was assayed and also was found to be comparable to that in other human cell lines (Appendix, Figure 3). These data suggest that, although stromal cells are negative in DNA-PKcs expression *in vivo*, they acquire a DNA-PKcs positive phenotype when grown in culture. This result is consistent with the observation that proliferating cells show higher levels of DNA-PKcs than resting cells (Kubota et al., 1998) and that stromal cells *in vivo* are terminally differentiated (non-dividing).

To determine if the corollary is true *in vivo*, that is if DNA-PKcs is low in resting, terminally differentiated breast tissue, we proposed to analyze the epithelial as well as the stromal cells in breast tissue sections from post-menopausal (vs. pre-menopausal) women where the major part of this gland should be at rest and in a non-proliferating state, i.e. the cells are terminally differentiated and have stopped dividing. When we studied the normal breast tissue sections from post-menopausal (age 65 and older) women, DNA-PKcs and Ku expression was found to be low in these tissues. However, these results were highly affected by the fact that not much of the mammary gland tissue was available for study in these tissue sections. Since by this stage these mammary glands must have gone through several cycles of pregnancy/lactation/involution, very few acinii were observed in typical tissue sections.

As a second approach to analyzing NHEJ expression in stromal cells from human breast tissues, we obtained samples of HNBFibs (human normal breast fibroblasts) from the laboratory of G. R. Cunha, University of California, Berkeley (Parmar et al., 2002). While analysis of these cells for NHEJ proteins by immunohistochemical and Western immunoblot analysis confirmed the absence of NHEJ protein expression, subsequent analyses to determine the methylation status of the *PRKDC* promoter suggested that the majority of the cells in these samples were of mouse origin rather than human. Mouse cell lines express 50 to 100 times less DNA-PKcs and Ku proteins than cultured human cells. That the HNBFibs were indeed primarily of murine origin was confirmed using species-specific PCR primers and by cytological analysis of metaphase chromosomes.

Okayasu et al. (2000) reported that DNA-PKcs and the Ku proteins were expressed at lower levels in mouse breast tissues than in other mouse tissues and further showed that BALB/c mice, which are 20 times more susceptible to low-level radiation-induced breast cancer than most other mouse strains (e.g. C57Black), expressed less DNA-PKcs protein than radiation resistant mouse strains. Furthermore, breast cancer susceptibility was associated with DNA double-strand break repair capacity, radiosensitivity and DNA-PKcs expression. More recently, two differences in the amino acid sequence of DNA-PKcs from BALB/c mice compared to C57Black mice were discovered (Yu et al., 2001). Although DNA-PK has been implicated in the cellular response to

ionizing radiation in mammalian cells (Muller et al., 1999), only very recently has an association been made between polymorphisms in NHEJ genes and breast cancer risk for humans (Fu et al., 2003). This group found two SNPs in Ku70 and XRCC4 were associated with breast cancer risk ($P < 0.05$) in women from northern Taiwan.

In collaboration with Dr. W. Kauffmann, Lineberger Cancer Center, University of North Carolina, we have begun an analysis of DNAs from EBV immortalized lymphocytes derived from 14 breast cancer patients participating in the Carolina Breast Cancer Program. The lymphocytes from seven of these individuals exhibit a chromosome breakage phenotype when exposed to ionizing radiation in the G2 phase of the cell cycle (see Scott et al., 1994), while the remaining seven do not. As indicated in the previous annual report, using an ABI 3100 automated DNA sequencer, both strands of PCR segments corresponding to each of the thirteen exons of the Ku70 gene from these 14 individuals were sequenced. No polymorphisms were identified that altered the amino acid sequence of Ku70, indicating that polymorphisms in Ku70 coding sequences are unlikely to be responsible for breast cancer-related radiation sensitivity phenotype or increased breast cancer risk in humans, at least within the population represented by these samples. A similar analysis of the 21 exons of Ku80 and 42 of the 86 exons of DNA-PKcs has since been made. Only one polymorphism was identified that affected the Ku80 amino acid sequence in the 28 alleles examined; a single nucleotide polymorphism was identified in exon 20 of one individual that changed the isolucine codon to valine. Likewise, in the 42 exons of DNA-PKcs that have been examined, only one coding single-nucleotide polymorphism, in exon 73, was identified. However, in the Ku80 gene, a high frequency deletion was identified in a 21 bp repeat sequence just upstream of the protein start site; only three of the 14 lines examined were homozygous for the full repeat sequence. Further analyses will be required to determine if the structure of this region of the Ku80 promoter affects Ku80 or KARP1 (a DNA damage-induced Ku80-related protein) expression or breast cancer susceptibility.

Through other resources, antibody reagents for analyzing posttranslational modifications to the p53 tumor suppressor protein have been developed. Analysis of p53 from oncogenic Ras transformed human IMR-90 normal human lung fibroblasts demonstrated phosphorylation on Ser33 and Ser46, indicative of phosphorylation by p38 MAPK. Overexpression of Wip1, a p53 induced protein phosphatase that inactivates p38 MAPK, decreased p53 phosphorylation at Ser33 and Ser46 and partially reversed Ras-induced senescence. Analysis of 64 human tumor lines showed that the Wip1 gene, *PPM1D*, at 17q22/23 was amplified in several breast tumor cell lines. Thirty-seven of 326 primary breast tumors (11.3%) also exhibited *PPM1D* amplification, suggesting that p53 inactivation through *PPM1D* amplification and overexpression may contribute to the development of human breast cancer. These studies, reported last year, are published in *Nature Genetics* (Bulavin et al., 2002). Subsequently, we have shown that mice deleted for the *PPM1D* gene are resistant to cancer induction, most likely as a consequence of the induction of high expression levels of $p19^{Ink4a}$. $p19^{Ink4a}$ inhibits cyclin D1/Cdk4 activity, which is necessary for cell cycle progression through G1. Our results (Bulavin et al., manuscript in preparation) suggest that inhibitors of Wip1 may be effective anti-cancer agents for a significant fraction of breast cancers. We also have shown that genistein, and isoflavinoid that is abundant in soy-derived products and has been linked to reduced rates of breast and prostate cancer in

populations who have a high soy-content in their diets, activated p53 and induced phosphorylation at multiple p53 sites in an ATM-dependent manner, while quercetin, a related isoflavinoid, activated p53 through both ATM- and ATR-dependent pathways (Ye et al., DNA Repair, submitted).

Key Research Accomplishments

- Rexamined expression of DNA-PKcs, Ku70/Ku80 and other NHEJ components, including XRCC4, DNA ligase IV and NBS1/MRE11 in epithelial cells from resting human breast tissue; each protein was expressed at similar, readily detectable levels.
- Examined expression of NHEJ components in stromal cells from resting and lactating human breast tissue; none were expressed.
- Identified two representative human breast stromal cell lines in which expression of DNA repair proteins could be examined to determine if reduced expression of NHEJ proteins is recapitulated.
- Studied expression of DNA-PKcs and Ku80 in cultured stromal cells as well as other human cell lines; expression was comparable to that found in other human cell lines.
- DNA-PK activity was assayed in cultured stromal cells, which also was found to be comparable to that found in other human cell lines.
- Showed no correlation between polymorphisms in Ku70 and radiation induced chromosome breakage phenotype.
- Determined an absence of polymorphisms in *KU70* from lymphocytes of breast cancer patients with and without a DNA breakage phenotype.
- Identified a high frequency length polymorphism in the *KU80* promoter region of cells from breast cancer patients.
- Identified *PPM1D*, the gene for the Wip1 phosphatase, as a gene frequently amplified in breast cancer patients with wildtype *TP53*.
- Determined that cells lacking *PPM1D* are resistant to cell transformation and that mice lacking *PPM1D* are resistant to oncogene-induced cancers in a model system.
- Showed that genestein, an isoflavinoid linked to breast and prostate cancer prevention, activates p53 in an ATM-dependent manner.

Reportable Outcomes

- **Differential expression of DNA double-strand break repair proteins in human breast cells** - M. Tawde, P. Friemuth, C. Anderson. Poster presented at the Era of Hope DoD Breast Cancer Research Program meeting held at Orlando, FL on September 25-28, 2002.
- **Differential expression of DNA double-strand break repair proteins in human breast cells.** M. Tawde, P. I. Freimuth, and C. W. Anderson (manuscript in preparation).
- **Amplification of *PPM1D* in human tumors abrogates p53 tumor suppressor activity.** D.V. Bulavin, O.N. Demidov, S. Saito, P. Kauraniemi, C. Phillips, S.A. Amundson, C. Ambrosino, G. Sauter, A.R. Nebreda, C.W. Anderson, A. Kallioniemi, A.J. Fornace, Jr., and E. Appella. *Nat Genet* **31**: 210 (2002).

- **Genistein induces multisite phosphorylation of human p53 via an ATM-dependent DNA damage response pathway**. R. Ye, A. A. Goodarzi, E. U. Kurz, S. Saito, Y. Higashimoto, M. F. Lavin, E. Appella, C. W. Anderson, and S. P. Lees-Miller. DNA Repair (submitted).
- **Deficiency of Wip1 phosphatase impairs mammary tumorigenesis.** D. V Bulavin, C. Phillips, B. Nannenga, L. A. Donehower, C. W. Anderson, E. Appella and A. J. Fornace, Jr. (manuscript in preparation).

Conclusions

Both resting as well as lactating breast tissue sections of normal women showed strong expression of DNA-PK, Ku70/80, XRCC-4, DNA Ligase IV as well as NBS1 and MRE11 in epithelial cells. The stromal cells in these tissue sections were found to be devoid of or to express low levels of each of these proteins. Although stromal cells in tissue sections showed low DNA-PK expression, when cultured *in vitro* they exhibited moderate expression levels and activity. Proliferating cells exhibit higher DNA-PK expression and activity compared with non-proliferating or resting cells.

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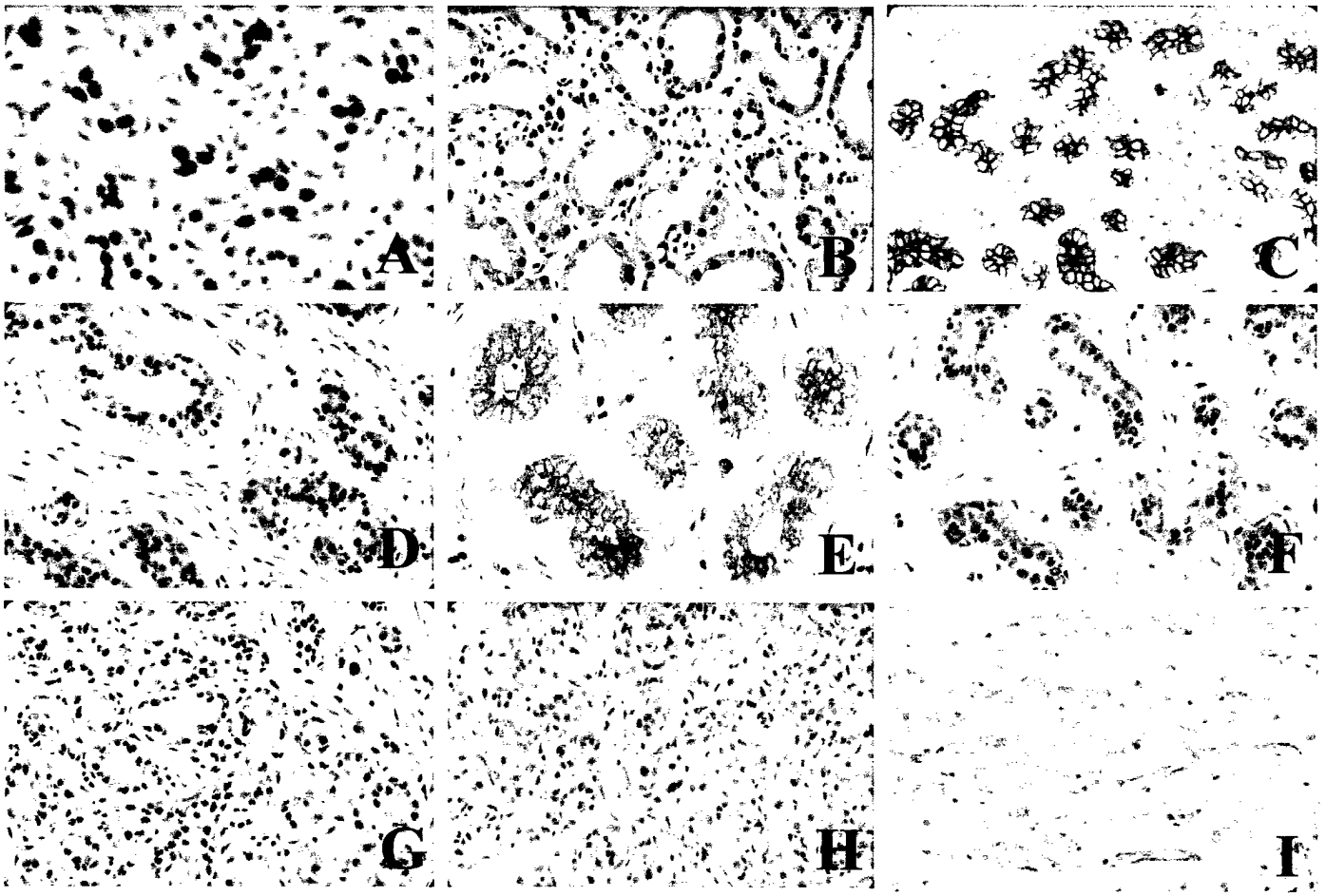

Figure 1. Expression of DNA-PK and other NHEJ DSB repair proteins in human breast tissue. A. DNA-PK in resting breast tissue; B. DNA-PK in lactating breast tissue; C. Ku80; D. XRCC4; E. DNA Ligase IV; F. Mre11; G. NBS1; H. PCNA; I. Mib(Ki67). Note that the stromal cells are negative. Methods were as described in Fig 2. The original magnification was 40x.

Appendix

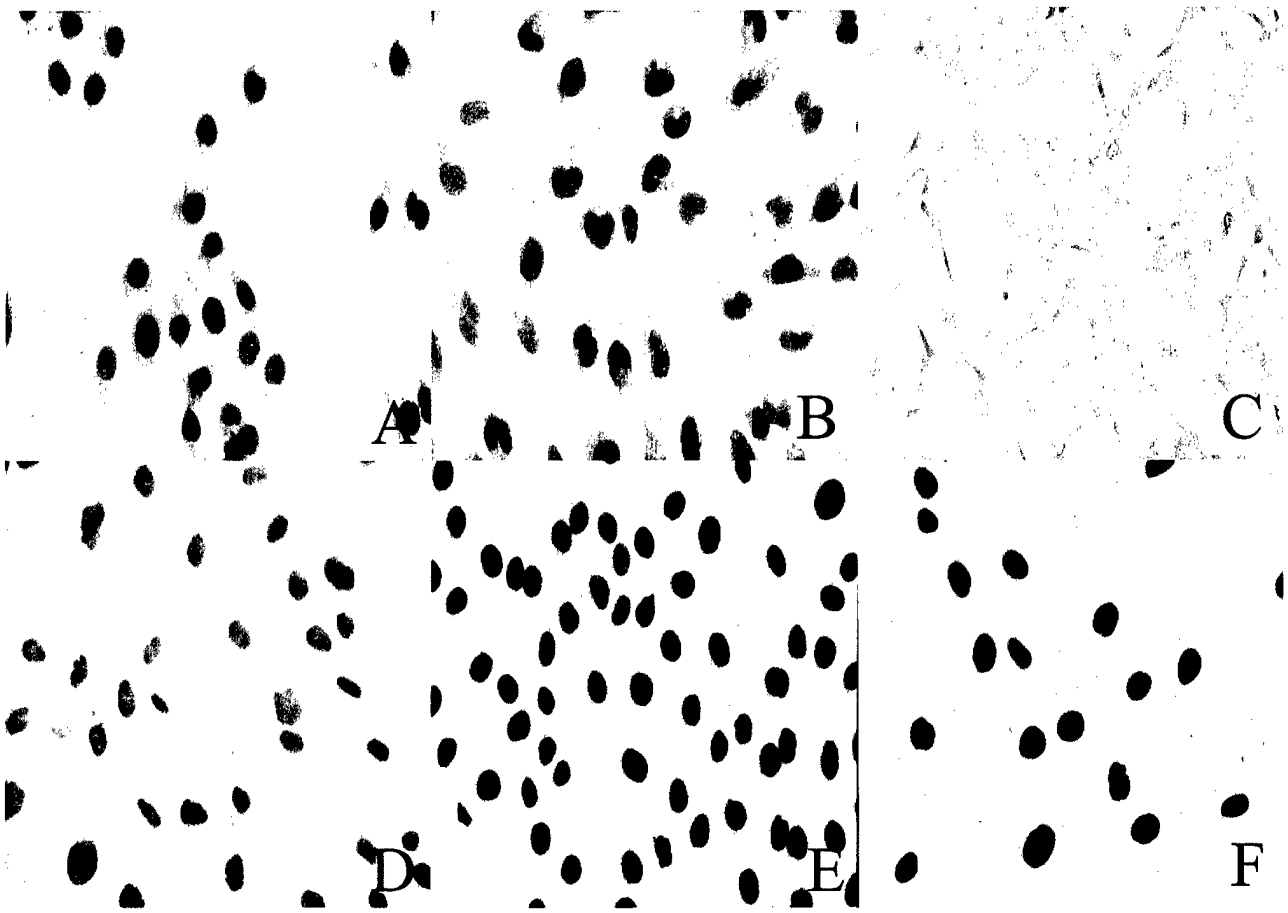

Figure 2. Expression of NHEJ repair proteins in cultured human cell lines. DNA-PK in A) HeLa; B) MO59K; C) MO59J and D) Hs 574.T cells. Ku-80 in E) HeLa and F) Hs 574.T cells. M059J cells lack DNA-PKcs protein due to a mutation in the *PRKDC* gene. Similar results were obtained for XRCC4, Ligase IV and the NBS1/Rad50/MRE11 complex proteins (data not shown). Tissues came from the routine pathology archives of the Department of Pathology, University Hospital at SUNY Stony Brook. After biopsy or resection, the tissues were fixed in 10% formalin for up to 18 h and processed for light microscopy by standard methods. Immunohistochemical staining was as described in Moll et al. (1999). Briefly, 4 μm paraffin sections were deparaffinized by microwaving sections in 100 mM citric acid buffer, pH 6.0 for 5 min, 6 times. Sections then were treated with 0.3% H_2O_2 / methanol to quench endogenous peroxidase activity. After blocking with 10% normal goat serum, sections were incubated at 4°C overnight with primary antibody in 2% bovine serum albumin/phosphate buffered saline. Biotinylated goat anti-mouse or goat anti-rabbit secondary antibodies and streptavidin/biotin complex were applied for 30 min each (ZYMED, San Francisco CA), followed by 8 min incubation in diaminobenzidine substrate and extensive washing. Sections were lightly counter-stained in hematoxylin and mounted under a coverslip. Sections were photographed with a Nikon photomicroscope. Original magnification was 40x.

Appendix

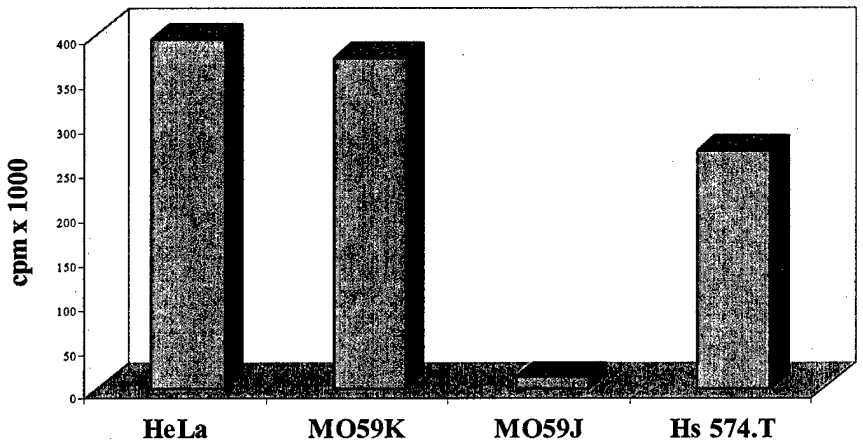

Figure 3. DNA-PK activity in different human cell lines. DNA-PK activity assayed in HeLa, MO59K, MO59J and Hs 574.T cells by the 'DNA pull-down' assay (Achari et al. 1999). Whole cell extracts were prepared by a modification of the method of Finnie et al.(1995). Briefly 10^7 cells were harvested, washed 3 times in PBS and the cell pellets were frozen at -80°C. Frozen cell pellets were resuspended in LSB pH 7.2 (10 mM HEPES, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA) containing 0.1 mM DTT and 0.2 mM PMSF and centrifuged at 1,000 x g for 10 min. The pellets were resuspended again with 2.5 x the packed cell volume of LSB and incubated on ice for 10 min to allow the cells to swell; they were then frozen by plunging them into liquid N_2. The cell extracts were quick thawed at 37°C in the presence of protease inhibitors (0.5 mM PMSF, 2 µg/ml each leupeptin and aprotinin), 200 µl were removed and adjusted to 0.5 M NaCl, 10 mM $MgCl_2$ by the addition of 22 µl 5 M NaCl, 100 mM $MgCl_2$, 5 mM DTT (Extraction Buffer, ExB). Extracts were incubated on ice for 3 min and then centrifuged at 10,000 x g for 3min at 4°C. Supernatants were removed and the pellets were extracted with 40 µl of 1/10 dilution of ExB (in 50 mM HEPES, pH 7.5) and pooled with the first supernatant. These whole cell extracts were then aliquoted, quick frozen and stored at -80°C until assayed. Protein concentrations were determined by the Bradford assay using BSA as a standard, and cell extracts equivalent to 0.5 mg protein were incubated with 50 µl preswollen dsDNA-cellulose beads (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C. The dsDNA-cellulose was washed three times with 1 ml of buffer A (25 mM HEPES, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, 0.5 mM EDTA, 0.25 mM EGTA, and 1 mM DTT) before it was resuspended in 50 µl distilled water; then assays were performed using the SignaTECT DNA-PK assay system (Promega, Madison, WI). Kinase reactions were conducted with 6-12 µl aliquots of the resuspended DNA-PK-absorbed cellulose beads and were performed in both the presence and absence of a biotinylated DNA-PK p53-derived substrate peptide. Terminated reactions were analyzed by spotting onto SAM^2 membranes, washing, and counting the incorporated radioactivity in a scintillation counter as per the manufacturer's instructions. All assays were performed in duplicate with at least three different extract preparations. The kinase activity was normalized to total protein content. Results shown are from a representative of three experiments.